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GENE CONTROLLING SHOOT BRANCHING IN PLANTS

Background of the Invention

Field of the Invention

This invention relates to a novel plant gene that encodes a protein which is involved in the control of plant growth and developmental hormones. More particularly the invention relates to a novel gene that encodes an enzyme involved in cytokinin metabolism and to methods for altering plant growth and development.

Background Technology

Modulation of plant hormone levels is important for plant growth and development, as well as for environmental adaptation. This regulation can be achieved via the regulation of hormone synthesis and/or metabolism.

Plants exhibit a wide range of aerial architecture. One of the characteristics that determines the overall plant structure is the shoot-branching pattern. Shoot branching is initiated by the formation of lateral meristem in the leaf axils. In some plant species the growth of the lateral meristem is inhibited by the primary floral shoot, a phenomenon generally known as apical dominance. Cytokinin and auxin are classical plant hormones that play a major

role in controlling such developmental process (1). (A bibliography is provided at the end of the written description.) Auxin represses the outgrowth of the lateral bud, whereas cytokinins stimulate growth. Imbalance in the two hormone levels causes growth defects. Besides the release of lateral buds from apical dominance, cytokinins also play several other important roles in plant growth and development. These include the delay of leaf senescence, inhibition of root growth and, together with auxin, promotion of cell division and shoot formation in tissue culture. This information is mostly derived from indirect evidence of exogenous application of cytokinins and from plants transformed with the bacterial cytokinin biosynthesis gene, IPT, which encodes isopentenyl transferase (2,3). Thus, knowledge of biosynthesis, metabolism, distribution, perception and signal transduction of cytokinins *in planta* is poorly understood. To this end, in the past few years, a number of cytokinin related mutants have been isolated from *Arabidopsis* (4-9). Several selection schemes have been employed to select for different classes of mutants, in an attempt to dissect mechanisms by which cytokinins regulate plant growth and development. Nevertheless, the molecular nature of the corresponding genes is largely unknown and the types of mutants isolated are mainly restricted by the design of the selection schemes.

Summary of the Invention

A gene, designated SPS, has been isolated from an *Arabidopsis* mutant, sps, which exhibits an abnormal shoot-branching pattern and developmental program. The *Arabidopsis* SPS gene has the nucleotide sequence shown in SEQ ID NO:1.

SPS has been shown to encode a cytochrome P450, which has been assigned as CYP79F1. Phenotypic abnormalities of the sps mutant and analysis of its hormone levels indicate that SPS encodes an enzyme involved in cytokinin metabolism. Accordingly, in one aspect, the present invention involves a method for altering growth or development of a plant containing a SPS gene which comprises modulating expression of the SPS gene.

In a further aspect, the invention provides novel plants transformed with the SPS gene operatively linked to a plant-active promoter. Also embodied within the invention are novel plants exhibiting enhanced cytokinin levels, which are due to suppression of the SPS gene. Suppression of SPS is achieved, for example, by transforming a plant with a nucleic acid encoding an SPS antisense or ribozyme operatively linked to a plant-active promoter. The plant-active promoters employed in these embodiments may be a constitutive promoter, an inducible promoter or a tissue specific promoter, as are well known to those skilled in plant molecular biology.

The CYP79 coding sequences are widely conserved among plants of different genera and species. Thus, the *Arabidopsis* SPS gene and primers and probes having

about 8 or more nucleotides derived from its sequence are useful in methods for preparing an isolated SPS gene of a plant of a different species.

These and other aspects and advantages of the invention in accordance with the scope of the claims will be apparent to those skilled in the art.

Brief Description of the Drawings

Figure 1 shows a wild-type and sps mutant of *Arabidopsis thalisana*.

Figure 2 shows the location of cytochrome P450 genes, as well as donor T-DNA and Ds insertion sites in sps alleles 1-5.

Detailed Description of the Invention

The SPS gene has been discovered through studies of *Arabidopsis* transposon insertion mutant designated supershoot ("sps"). The sps mutant was selected from a collection of gene trap Ds insertion lines (10) in *Arabidopsis thaliana* ecoptype Wassilewskija (Ws).

The phentotypic abnormalities and elevated cytokinin levels in the sps mutant indicate that the mutant is defective in the regulation of cytokinin levels. It was postulated that the Ds element had transposed to an insertion site of a gene involved in cytokinin metabolism, thereby inactivating the gene to cause cytokinin accumulation.

Because the putative gene was tagged with the Ds element, a fragment of genomic DNA flanking the Ds element from a mutant plant was amplified by TAIL-PCR

(17) for detailed analysis. A search of the *Arabidopsis thaliana* genomic database revealed that the flanking sequences were identical to the genomic sequences from chromosome 1, GenBank GI 4887257; GenBank accession number AC 006341. The donor T-DNA locus used in the transposon-tagging experiment was also found to be within the same clone. A comparative study between the positions of the donor and re-insertion sites indicated that, in this mutant (designated sps-1 allele, because, as described below, additional alleles also have been found), the element transposed 8.5 kb away from the donor site into the coding region of a putative cytochrome P450, assigned previously as CYP79F1 (AC006341, 63874..66127). The PCR fragment was then used as a probe to screen *Arabidopsis thaliana* leaves and a stem cDNA library (18). The longest SPS cDNA clone isolated from the screen was 1.38 kb long. This cDNA was determined to be a partial clone, truncated at the 5' end. Available genomic and EST sequences corresponding to the SPS gene (19) predicted that the start codon of the open reading frame was located 442 bp before the beginning of the isolated cDNA. This prediction was supported by the information derived from the SPS closely related gene, which exhibits 89% sequence identity to the SPS gene (see below). The sequence of the full length SPS cDNA was reconstituted. It is 1.8 kb long and has the sequence shown in SEQ ID NO:1. The SPS gene is predicted to encode 537 amino acids, with a calculated molecular mass of 61.5 kD.

The number of closely related sequences within the CYP79F subfamily was estimated by genomic southern blot

analysis (20). In the *Arabidopsis* genome, only two fragments hybridized to the SPS probe under low stringency, suggesting that there are only two related members in this sub-family. A search of the *Arabidopsis* database revealed that the closely related sequence locates just next to the SPS gene and has been assigned as CYP79F2 (AC006341, 60246..62290). Locations of the two Cytochrome P450s as well as the donor T-DNA are shown in Fig. 2. To date, 13 members in the CYP79 family have been identified in plant (seven members from *Arabidopsis*; one from *Sorghum bicolor*; one from *Sinapis alba*; two from *Manihot esculenta*; two from *Triglochin maritima*). Among these 13 members, 6 members have been studied in more detail. CYP79A1 from *Sorghum bicolor* (21), CYP79B1 from *Sinapsis alba* (22), CYP79E1 and CYP79E2 (43) from *Triglochin maritima* are shown to catalyze the conversion of tyrosine to *p*-hydroxyphenylacetaldoxime in the biosynthesis of plant secondary metabolites, the cyanogenic glucosides. CYP79B2 and CYP79B3 from *Arbidopsis* are shown to catalyze the conversion of tryptophan to indole-3-acetaldoxime, a precursor to IAA and indole glucosinolate (44).

To confirm whether CYP79F1 corresponds to the sps mutation, several additional sps alleles caused by independent Ds insertion were isolated. These mutant alleles were readily obtained due to the proximity of the donor T-DNA and the SPS gene. Four more sps alleles, designated sps-2 to sps-5, were isolated from independent insertion lines. Each allele exhibited overall phenotype similar to the sps-1. Genomic Southern blot analysis confirmed that each line

contained only a single Ds element in the genome. The Ds-flanking DNA fragment from each allele was isolated and analyzed. All of these alleles contained Ds insertions within the coding region of the CYP79F1 gene. The positions of the Ds elements that disrupt the gene function in the 5 mutant alleles are shown in Fig 2. Taken together, these results indicate that SPS gene is tagged with Ds element.

There are several sequence motifs conserved among members of the cytochrome P450 and are reported to be important for the protein function. These include the heme-binding domain essential for catalysis, N-terminus hydrophobic region essential for membrane association and a proline/glycine-rich region for proper protein assembly (23, 24). The heme-binding domain of the CYP79 family diverges from other P450s but is conserved among members in the same family. A CYP79 family specific heme binding consensus sequence is defined as SFSTG(K/R)RGC(A/I)A (22). This unique sequence was also conserved in the SPS gene. The N-terminal hydrophobic region and the proline/glycine-rich region are also present.

Control or modification of plant growth and development can be achieved by modulating expression of the SPS gene. Cytokinin levels can be increased by suppressing expression of the gene. Such increased levels of cytokinins enhance lateral shoot formation (27, 28). In contrast, over-expression of the SPS gene using sense constructs under the control of strong promoters suppress lateral shoot formation.

Furthermore, the aerial architecture of a plant can be altered within a defined region by controlling

the location and/or timing of expression of the SPS gene. For example, the modification that leads to only an increase of lateral branches may be achieved by reducing the SPS expression level at the site of bud initiation, with the use of specifically expressed or tissue-specific promoters. Such promoters are known and can be readily isolated by various technologies such as gene trapping (25).

Suppression of SPS expression can be accomplished in a variety of ways known in the art. Antisense technology can be conveniently used. To accomplish antisense expression, a nucleic acid segment from the SPS gene is cloned and operably linked to a promoter such that the antisense strand of RNA will be transcribed. The expression cassette is then transformed into plants and the antisense strand of RNA is produced. In plant cells, it has been suggested that antisense RNA inhibits gene expression by preventing the accumulation of mRNA which encodes the gene of interest (29, 30).

The nucleic acid segment to be introduced generally will be substantially identical to at least a portion of the SPS gene. The sequence, however, need not be perfectly identical to inhibit expression. For antisense suppression, the introduced sequence need not be full length relative to either the primary transcription product or fully processed mRNA. Generally, higher homology can be used to compensate for the use of a shorter sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments may be equally effective. Normally, a sequence of between

about 30 or 40 nucleotides and about full length may be used, though a sequence of at least about 100 nucleotides is preferred, a sequence of at least about 200 nucleotides is more preferred, and a sequence of at least about 400 nucleotides is especially preferred.

Catalytic RNA molecules or ribozymes can also be used to inhibit expression of the SPS gene. It is possible to design ribozymes that specifically pair with virtually any target RNA and cleave the phosphodiester backbone at a specific location, thereby functionally inactivating the target RNA. In carrying out this cleavage, the ribozyme is not itself altered, and is thus capable of recycling and cleaving other molecules, making it a true enzyme. The inclusion of ribozyme sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs.

A number of classes of ribozymes have been described (31). One class of ribozymes is derived from a number of small circular RNAs which are capable of self-cleavage and replication in plants. The RNAs replicate either alone (viroid RNAs) or with a helper virus (satellite RNAs). Examples include RNAs from avocado sunblotch viroid and the satellite RNAs from tobacco ringspot virus, lucerne transient streak virus, velvet tobacco mottle virus, Salanum nodiflorum mottle virus and subterranean clover mottle virus.

Enhancement of SPS expression may be accomplished by transforming a plant with an SPS gene operatively linked to a plant-active promoter. Techniques for incorporating DNA into a plant cell so as to achieve expression in the cell are well known. These

techniques are applicable to incorporation of the SPS gene and to incorporation of antisense or ribozyme constructs.

Generally, such techniques involve inserting the nucleic acid into a DNA expression vector. Such vector advantageously contains the necessary elements for the transcription and translation of the inserted protein coding sequences and one or more marker sequences to facilitate selection of transformed cells or plants.

A number of plant-active promoters are known in the art and may be used to effect expression of the desired nucleic acid sequences. Suitable promoters include, for example, the nos promotor, the small subunit chlorophyll A/B binding polypeptide, the 35S promotor of cauliflower mosaic virus, as well as promoters isolated from the SPS gene. The promoter may be isolated from the type of plant to be transformed. The 35S or actin promoters may also be used for isolated cDNA clones. These are also useful to test overexpression of the gene. Alteration of the *SPS* gene expression in defined regions of the plant could be achieved by using specifically expressed promoters. Such promoters can be readily isolated by various technologies such as gene trapping (25). Alternatively, several inducible promoters, such as GVG, GVGEc, ER-C1 systems, have been described (45). These inducible promoters can be used to turn on or turn off expression of a transgene.

Once the nucleic acid has been cloned into an expression vector, it is ready to be transformed into a plant cell. The term plant cell includes any cell derived from a plant; this includes undifferentiated

tissues such as callus and suspension cultures, as well as plant seeds, pollen or plant embryos. Plant tissues suitable for transformation include leaf tissues, root tissues, meristems, protoplasts, hypocotyls cotyledons, scutellum, shoot apex, root, immature embryo, pollen, and anther.

One technique of transforming plants with the SPS gene in accordance with the present invention is by contacting tissue of such plants with an inoculum of bacteria transformed with a vector comprising a nucleic acid in accordance with the present invention. Generally, this procedure involves inoculating the plant tissue with a suspension of bacteria and incubating the tissue for 48 to 72 hours on regeneration medium without antibiotics at 25-28°C.

Bacteria from the genus *Agrobacterium* can be utilized to transform plant cells. Suitable species of such bacterium include *Agrobacterium tumefaciens* and *Agrobacterium rhizogens*. *Agrobacterium tumefaciens* (e.g., strains LBA4404 or EHA105) is particularly useful due to its well-known ability to transform plants.

Another approach to transforming plant cells with the nucleic acid of this invention involves propelling inert or biologically active particles at plant cells. This technique is disclosed in U.S. Pat. Nos. 4,945,050, 5,036,006 and 5,100,792 all to Sanford et. al., which are hereby incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior

thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the nucleic acid of interest. Biologically active particles (e.g., dried yeast cells, dried bacterium or a bacteriophage, each containing DNA sought to be introduced) can also be propelled into a plant cell tissue.

Another method of transforming plant cells is the electroporation method. This method involves mixing the protoplasts and the desired nucleic acid and forming holes in the cell membranes by electric pulse so as to introduce the DNA in the cells, thereby transforming the cells. This method currently has high reproducibility and various genes have been introduced into monocotyledons, especially rice plants by this method (32-34).

Similar to the electroporation method is a method in which the desired gene and protoplasts are mixed and the mixture is treated with PEG, thereby introducing the gene into the protoplasts. This method is different from the electroporation method in that polyethylene glycol ("PEG") is used instead of the electric pulse (35-37).

Other methods include 1)culturing seeds or embryos with nucleic acids (38); 2) treatment of pollen tube (39); 3) liposome method (40, 41); and 4) the microinjection method (42).

Known methods for regenerating plants from transformed plant cells may be used in preparing transgenic plants of the present invention. Generally, explants, callus tissues or suspension cultures can be exposed to the appropriate chemical environment (e.g.,

cytokinin and auxin) so the newly grown cells can differentiate and give rise to embryos which then regenerate into roots and shoots.

Sequences of cytochrome P450 family of proteins, including CYP79F1, are conserved among a wide variety of plant genera and species (21, 22, 43, 44). Accordingly, the *Arabidopsis* SPS gene described herein may conveniently be used directly for affecting growth and development of plants, or may be used to prepare SPS genes and gene constructs (including antisense constructs) from other species. Primers and probes useful for identifying such SPS genes, optionally labeled with one or more detectable nucleotides, consist of a nucleic acid having a sequence that is the same as or is complementary to a sequence of any 8 or more, preferably 13 or more, contiguous nucleotides of SEQ ID NO:1. As used herein, "same as" refers to the sequence, recognizing that RNA sequences contain ribonucleotides that correspond to deoxyribonucleotides of DNA. Such primers and probes can be used in a method for isolating an SPS gene by, for example, amplification and/or probing, as is well known in the art.

The invention is further illustrated by the following examples, which are not intended to be limiting.

Example 1

Characterization of SPS Mutant

Morphology

The phenotype of sps mutants is not detectable in young seedlings, but pleiotropic developmental defects can be noticed after plants produce adult leaves. See Figure 1. In sps mutant plants, leaf shape is more serrate and leaf vasculature is less well developed relative to wild type plants. In *Arabidopsis*, the pattern of vasculature in the cotyledon is simple with only a central vein and two or three interconnecting side branches. The pattern becomes more complex in rosette leaves, with the greatest complexity seen in the late adult leaves. In sps mutants, the pattern of the vasculature in the rosette leaves is much less complex than that in wild type leaves. In addition, the veins of the adult leaves become strikingly prominent, appearing dark green, compared to wild type. When chlorophyll contents were determined in mature leaves, the mutant plants also contain higher level of chlorophyll (11). The severity of these vegetative phenotypes varies depending on growth conditions. The phenotypes can be significantly delayed by growing plants on the synthetic medium.

The phenotypic defects in the sps mutants become more pronounced after the plants start to flower. The sps mutant has the same rate of leaf initiation during vegetative stage and the timing of the transition from vegetative to reproductive phase does not differ from wild type. The sps floral shoot has reduced internode elongation and displays a loss of apical dominance (Fig 1A). In wild type *Arabidopsis* plants, the number of lateral floral shoots developing from rosette leaves varies, commonly less than 5, depending on the ecotype. However, mutant plants, which also display a delay of

senescence, continue to produce lateral floral shoots from both the axials of rosette and cauline leaves resulting in several hundred shoots per plant after 4-5 months (Fig 1B). Sps mutants display a reduced number of floral organs and are defective in floral development. Petals are usually reduced or missing. The number of sepals and stamens are also reduced in some flowers. Anthers do not usually release pollen grains and the stigmas remain underdeveloped. Consequently, the mutant has a very low seed production.

Cytokinin Levels

The physiological changes in the aerial part of the sps mutant plants are strikingly similar to the effects of cytokinin caused by exogenous application of hormone and is characteristic of transgenic plants exhibiting cytokinin overproduction (3). These physiological changes include the release of lateral buds from apical dominance, the increase in bud initiation, the delay of leaf senescence as well as the increase in chlorophyll content. Based on these observations, levels of free zeatin, which is the major active cytokinin in *Arabidopsis* (5,6) were determined. The sps mutant has an average of three times more active zeatin than wild type (12). The phenotype of sps suggests that increased cytokinin level is likely to be the primary defect responsible for these changes. Transgenic plants expressing the bacterial IPT gene, which leads to the morphological changes, were mostly reported to exhibit very high cytokinin levels, 50 folds or more (13-15). This dramatic increase in cytokinin is now considered to be non-physiological.

It has been shown that relatively minor changes in cytokinin level, two to sevenfold, are sufficient to cause the same developmental alterations (15,16). Results from sps mutant studies indicated that, indeed, small fluctuations of cytokinin levels can induce developmental changes *in planta*. The *Arabidopsis* mutant altered meristem program (ampl) has also been shown to have elevated level of endogenous cytokinin (5,6). Even though sps and ampl have loss of apical dominance, reduced fertility and delay senescence, they differ significantly with respect to other developmental programs. Other ampl phenotypes, such as polycotyly, faster rate of leaf initiation and abnormal phyllotaxy, have not been observed in sps, or in transgenic plants conferring cytokinin overproduction. On the other hand, the reduced number of floral organs and the characteristic of the leaf veins, appearing dark green, found in sps mutants are not observed in the ampl mutant. To date, there is no molecular information on the structure and function of the AMP1 gene.

SPS Expression Pattern Monitored by Gene Trap Ds Element

Information about the gene action was derived from the study of temporal and spatial expression of the gene in the sps-2 allele. In this allele, a GUS reporter gene was incorporated between the ends of the Ds element. Expression of the GUS reporter was controlled by the natural SPS promoter in these constructs (25). The expression pattern of SPS as exhibited by the GUS reporter correlates well with the morphological abnormalities observed in the aerial

parts. Expression is first detected in the seedling 4-5 days after germination at the branching area of the vascular tissue that lies at the base of the shoot apical meristem. A few days later, the expression level is more intense and found in the vascular tissue in both the hypocotyl and the cotyledons. Expression pattern in the leaf vasculature appears to be developmentaly regulated. It is first observed only in the mid vein in the young leaves and then, when the leaves are more mature, in the finer veins. Expression of SPS gene is restricted solely to the aerial part of the plant which continues from the vegetative phase to reproductive phase. In the floral shoots, it expresses in the vascular tissue of the stem and cauline leaves, with the strongest intensity at the base of the cauline leaves, and at the receptacle of the flower and silique. No SPS expression is observed in the root system either in the seedling stage or in the adult plant. These results suggest that SPS localizes the modulation of cytokinin levels, which in turn control development in defined regions of the plant. SPS expression levels observed in mutant plants are much stronger than that observed in heterozygous plants exhibiting wild type phenotype. These data imply that expression of SPS is controlled by a feedback regulation mechanism that can enhance expression level upon disruption of the gene. Such a regulation may reflect the ability of the plants to fine tune the level of hormone in response to the fluctuations of internal or external stimuli.

The question of how plants modulate cytokinin spatially and temporally can be deciphered from the

analysis of SPS gene expression pattern. This question was difficult to address in the past, due to the fact that plant hormones are present in minuscule amounts in most plant tissues. It has been shown unambiguously that cytokinin is a key factor in promoting bud growth, whereas auxin has an inhibition effect. Therefore the outcome appears to depend on the ratio of the two hormones. Despite this well-established concept of hormone interaction, the mechanism of apical dominance remains unclear. The results obtained from sps strongly suggest that the plant maintains apical dominance in part by attenuating active cytokinin in the meristematic region to the level that it can inhibit the release of bud growth, as well as de novo bud formation.

Example 2

Experimental Description of *SPS* Cloning

To isolate DNA immediately flanking the *Ds*, about 10 ng genomic DNA from leaf tissue of the mutant plants was used for amplification by TAIL-PCR as previously described (17). The amplified fragments carrying the flanking genomic sequences were isolated by gel electrophoresis and sequenced using standard protocols (20). The PCR amplified fragments were then labeled with ^{32}P-dCTP and used as a probe to screen a cDNA library made from *Arabidopsis* leaves and stems (Clontech) as described (20). Phages that hybridized to the PCR fragment were purified and characterized (20 and instructions from manufacturer Clontech). The

inserts were then subcloned into the BLUESCRIPT plasmid (Stratagene) for detailed analysis in accordance with manufacturer's instructions. Plasmids carrying the inserts were sequenced using an automated sequencer (Perkin Elmer AMI 377) and the full length sequence was assembled using the DNA STAR program.

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